Synthesis of Thieno[2,3]-pyrimidines as Candidate Antileishmanial Agents

Undergraduate Honors Thesis

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Introduction

Leishmaniasis is one of several parasitic diseases which contribute to the high rate of mortality in developing countries. In addition, several hundreds of people are now dually infected with HIV and leishmaniasis, making both infections harder to treat. Leishmaniasis has also been an epidemic in countries such as Sudan, India and Afghanistan. Current antileishmanial drugs have severe limitations such as toxicity, the development of resistance and administration by injection [1].

The research currently being done to find better antileishmanial drugs is very limited due to the limited number of resources the pharmaceutical industry is willing to commit to such work. The lack of funding available to such research is due to economics: People of developing countries simply will not be able to pay for the drugs developed by pharmaceutical companies as a result of research in this area. Consequently, research focused on finding new drugs against these parasitic diseases becomes the responsibility of academia and other non-profit organizations.

The current research in this area focuses on developing drug treatment that is more affordable and more easily administered than the current treatment options which have many limitations. The goal of the research is to develop drug treatment which will target a critical process in the parasites, but will not be toxic to the mammalian host. Concurrently, Dr. Werbovetz's lab has been synthesizing and evaluating the effectiveness of dinitroaniline sulfonamides to achieve this goal which are shown in Figure 1 [2,3].

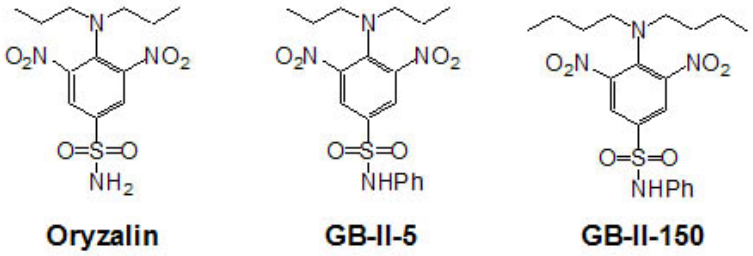


Figure 1. Dinitroaniline sulfonamides studied in Dr. Werbovetz's lab.

This project mirrors what has already been done with the dinitroaniline sulfonamides, but using a new class of molecules as antiparasitic drug candidates which was identified through previous random screening of a compound library. The lead antileishmanial compounds which were identified at Walter Reed are shown in Figure 2.

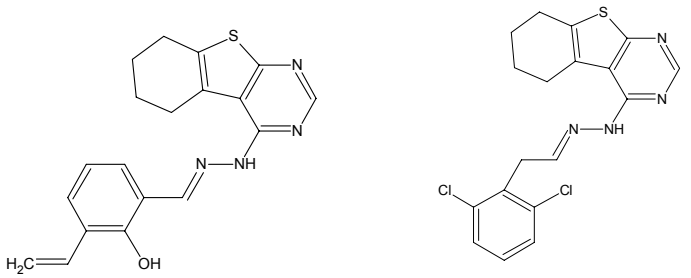


Figure 2. Antileishmanial lead compounds identified at Walter Reed.

The first portion of my project focused on the synthesis of 4-chloro-2-(chloromethyl)-6,7,8,9-tetrahydro-5*H*-indeno[2,1-*d*]pyrimidine, (**4**), to which different functional groups can be added to create a series of new molecules with the hope of preparing compounds with good antiparasitic activity. Once **4** had been synthesized, 9 novel compounds each with different R groups were synthesized (see Scheme 1). These compounds were then tested for their effectiveness in selectively inhibiting the growth of *Leishmania* parasites and not harming mammalian cells.

Materials & Methods

Chemistry
General Methods.

Unless otherwise indicated, all reagents and solvents were from Aldrich and were used without further purification. The melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 250 and 300 MHz NMR

spectrometers. Thin-layer chromatography was conducted on precoated TLC plates from E. Merck.

2-Amino-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile (3). Compound **3** was prepared according to the method of Gewald et al. [4]: yield, 31.5%; mp =142 °C; ^{1}H NMR ($CDCl_3$) δ 1.76-1.86 (m, 4H), 2.50-2.55 (m, 4H), 4.61 (bs, 2H).

4-Chloro-2-(chloromethyl)-6,7,8,9-tetrahydro-5*H*-indeno[2,1-*d*]pyrimidine (4). A suspension of **3** (4.00 g, 0.0225 mol), chloroacetonitrile (3.39 g, 0.0450 mol) and dioxane (22.5 ml, 0.264 mol) was stirred for eight hours in the presence of dry HCl gas at 5-10°C. The solution was then neutralized upon which a solid precipitated out. The crude product **4** was re-crystallized from hexanes and yielded 1.5 g (23%) of **4** as light yellow crystals: mp 92 °C; ^{1}H NMR ($CDCl_3$) δ 1.90-1.98 (m, 4H), 2.89-2.92 (m, 2H), 3.10-3.13 (m, 2H), 4.78 (s, 2H); HRMS (ESI) calcd for $C_{11}H_{10}Cl_2N_2NaS$ $(M + Na)^+$ 294.9839, measured $(M + Na)^+$ 294.9839.

General Methods for 5a-i. A suspension of **4** (1 equiv), the desired amine (10 equiv) and methanol (10 ml) were combined in a round bottom flask and allowed to stir at reflux for three hours. **5a-i** were purified using column chromatography performed on basic alumina gel and the products were eluted using 9:1 hexanes:ethanol.

***N*-Ethyl-2-[(ethylamino)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-amine (5b).** Yield 11.3 mg (5.7%) ^{1}H NMR ($CDCl_3$) δ 1.19-1.28 (m, 6H), 1.91 (m, 5H), 2.75-2.90 (m, 6H), 3.60-3.64 (m, 2H), 4.17 (s, 2H), 5.13 (bs, 1H); ^{13}C NMR ($CDCl_3$) δ 15.01, 22.62, 25.39, 26.38, 30.90, 35.82, 47.73, 54.94, 60.04, 114.08, 125.07, 131.97, 157.16, 163.10, 165.96.

***N*-Propyl-2-[(propylamino)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-amine (5c).** Yield 37 mg (8.4%), ^{1}H NMR ($CDCl_3$) δ 0.84 (t, *J* = 7.5 Hz, 3H), 0.99 (t, *J* = 7.5 Hz, 3H), 1.60-1.70 (m, 4H), 1.88-1.94 (m, 5H), 2.77-2.90 (m, 6H),

3.53-3.59 (m, 2H), 4.17 (s, 2H), 5.21 (t, *J* = 5.4 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.54, 11.97, 20.91, 22.62, 22.68, 22.86, 25.39, 26.40, 42.62, 55.87, 60.63, 114.07, 125.05, 131.88, 157.29, 163.25, 165.98; HRMS (ESI) calcd for $C_{17}H_{27}N_4S$ $(M + H)^+$ 319.1956, measured $(M + H)^+$ 319.1955.

***N*-Butyl-2-[(butylamino)methyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-amine (5d).** Yield 8 mg (3.4%) ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 7.4 Hz, 3H), 0.97 (t, *J* = 7.4 Hz, 3H), 1.25-1.68 (m, 8H), 1.88-1.94 (m, 5H), 2.77-2.90 (m, 6H), 3.57-3.63 (m, 2H), 4.16 (s, 2H), 5.17 (t, *J* = 5.2 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 13.90, 14.11, 20.25, 20.74, 22.62, 22.68, 25.39, 26.38, 29.77, 31.78, 40.61, 53.54, 60.56, 114.07, 125.04, 131.91, 157.28, 162.61, 165.91.

2-(Anilinomethyl)-*N*-phenyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-amine (5e). Yield 75 mg (28.1%) ^{1}H NMR ($CDCl_3$) δ 1.94-2.03 (m, 4H), 2.86-2.90 (m, 2H), 3.06-3.10 (m, 2H), 4.48 (s, 2H), 5.12 (bs, 1H), 6.70-6.76 (m, 3H), 7.13-7.25 (m, 4H), 7.37-7.42 (m, 2H), 7.62-7.65 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 22.44, 22.56, 25.53, 26.43, 49.39, 113.18, 115.00, 117.26, 121.11, 123.87, 124.69, 128.95, 129.21, 134.06, 138.49, 148.05, 154.66, 160.86, 166.81.

Biological Evaluation

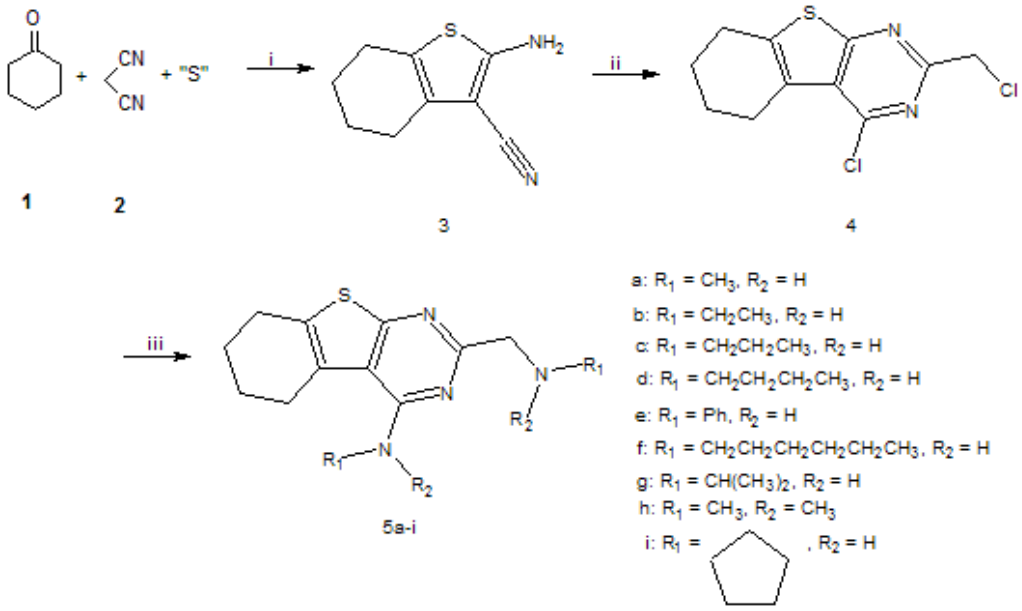
Testing against *L. donovani* axenic amastigotes. This assay was conducted as described previously by Havens et al. [5]. Briefly, *L. donovani* axenic amastigotes were seeded at 10^6 parasites/mL in 96-well flat-bottom plates with serial dilutions of the compound of interest. Plates were incubated for three days at 37°C, then the CellTiter reagent (Promega, Inc.) was added to assess cell viability. The absorbance of each well was measured after a four hour incubation to determine the concentration which inhibited growth of the parasites by 50% compared with the control.

Testing against Vero cells. The toxicity of the compounds against Vero cells was carried out as outlined by Salem and Werbovetz [6]. Briefly, Vero cells were plated at 10^4 cells/mL with serial dilutions of compounds for three days at 37°C, then viability was assessed using the CellTiter Reagent as described above.

Results & Discussion

Chemistry

Thieno[2,3]-pyrimidines were prepared as outlined in Scheme 1.



Scheme 1. Reagents and conditions: (i) diethylamine, ethanol, 40 °C [5,6]; (ii) Dry HCl gas, chloroacetonitrile, dioxane 5-10 °C for 8 hours [4,7]; (iii) NHR_1R_2, methanol, reflux

Cyclohexanone, malononitrile and elemental sulfur were combined the presence of ethanol and diethylamine. This reaction mixture was stirred at 40 °C for 24 hours. The workup was carried out using ethanol to re-crystallize the crude solid that formed during the reaction. This provided 2-amino-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile

(3) in approximately 40% yield. Compound **3**, chloroacetonitrile and dioxane were then combined and allowed to stir in the presence of dry HCl gas at 5-10 °C for eight hours. The reaction mixture was then basified with sodium bicarbonate. The solid was then re-crystallized in hexane resulting in **4**, my molecular building block. Compound **4** was then combined with the amines indicated in Scheme 1 in the presence of methanol and allowed to stir for four hours.

A homologation series was formed by synthesizing five compounds with alkylamine chain lengths varying from one to six carbons. A compound with phenyl substituents, a compound with a branched alkyl chain, a compound containing tertiary amines and a compound with cyclopentyl substituents completed the series as shown in Scheme 1.

One of the original aims of the project was to synthesize mono-addition products so that compounds bearing two different amine substitutions could be tested on the parasites, resulting in a more complete structure activity relationship. Thin-Layer Chromatography was used to determine when a mono-addition product versus the di-addition product was formed. Using methanol as the solvent for the reaction between **4** and the amines, the spots for these two products were so close in R_f that they almost appeared as one spot on the TLC plate. The reaction was run in various solvents to see if this would change the reactivity of compound **4** with amines. The solvents tested included dichloromethane, chloroform, ethanol, DMF and pyridine. In none of these solvents did a mono addition product form separately from the di addition product. The amine used as the nucleophile to test the effect of these solvents on the reaction was diethylamine since the reaction seemed to run the slowest according to TLC. Additionally, the reaction was run at 0 °C using propylamine as the nucleophile; however, in this case, no reaction was observed. Room temperature was the lowest temperature at which the reaction between **4** and propylamine would occur at a

reasonable rate. Consequently, running the reaction at 0 °C also did not allow for mono-addition.

The yields of the target compounds were very low with **5a-d** all being below 10%. One of the reasons for this was the purification process. The crude yields were often greater than 50%, however, much of this material was lost on the alumna column. The yields of compounds **5e-i** was more than twice that of **5a-d**. There are two reasons for this. First, as more targets compounds were prepared, the technique improved and consequently, the yields did as well. Second, compounds **5e-i** were prepared using higher molecular weight amines which were less likely to evaporate during the reaction than the lower molecular weight amines used earlier.

Biological Testing

Compounds **5a-i** have been tested for their effectiveness in inhibiting the growth of *L. donovani* axenic amastigotes and **5a-d** have been evaluated for toxicity against African Green Monkey kidney (Vero) cells. These results are shown in Table 1.

Table 1. Activity of Compounds Against *L. donovani* and Vero Cells In Vitro

Compound	Average IC_{50} vs *L. donovani* (μM)	IC_{50} vs Vero Cells (μM)
Pentamidine	1.7 ± 0.47[a]	
5a	2.4 ± 0.52[a]	12
5b	5.0 ± 0.86[a]	6.0
5c	3.8 ± 1.3[a]	5.3
5d	3.9 ± 0.52[a]	6.1
5e	>100	NT[c]
5f	16-22[b]	NT
5g	9.7-21[b]	NT
5h	28-58[b]	NT
5i	>50	NT

[a]Results expressed as the mean ± standard deviation of three independent experiments
[b]Results expressed as the range of two independent experiments
[c]Not tested

The best anti-parasitic activity was seen in the compounds containing short alkyl amine substitutions. The hexylamine compound **5f** and the isopropyl amine compound **5g**

showed lower antileishmanial activity, indicating that longer alkyl chains or branched chains lead to a loss of activity. Addition of aromatic groups and cyclic groups essentially eliminated all antiparasitic activity.

In conclusion, this work illustrates that the short alkyl amine compounds have the best antiparasitic activity. Moreover, the methylamine compound **5a** exhibited the best selectivity ratio of all the compounds. Further research to identify the antiparasitic target of **5a** as well as analogs to promote more specificity and potency may prove to be a successful approach in the search for improved antileishmanial therapy.

Acknowledgements. I would like thank Todd Barszcz and Jeff Capers for performing the assays against *L. donovani* and Vero cells, respectively.

References:

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Appendix A. NMR Spectra for **5b-e**. Mass Spectrometry for **5c**.

Figure A1. Proton NMR Spectrum for **5b**.

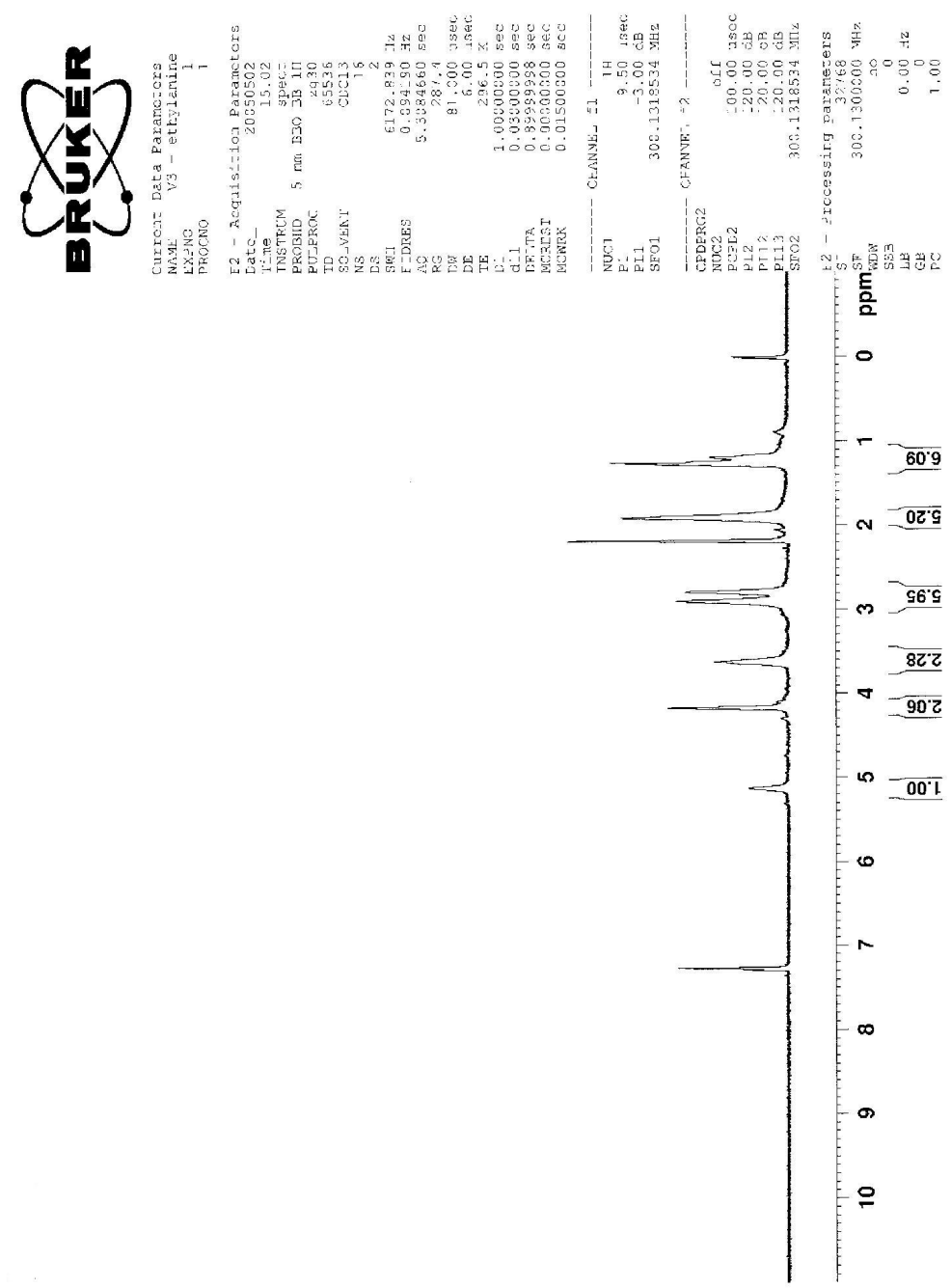

Figure A2a. Proton NMR Spectrum for **5c**.

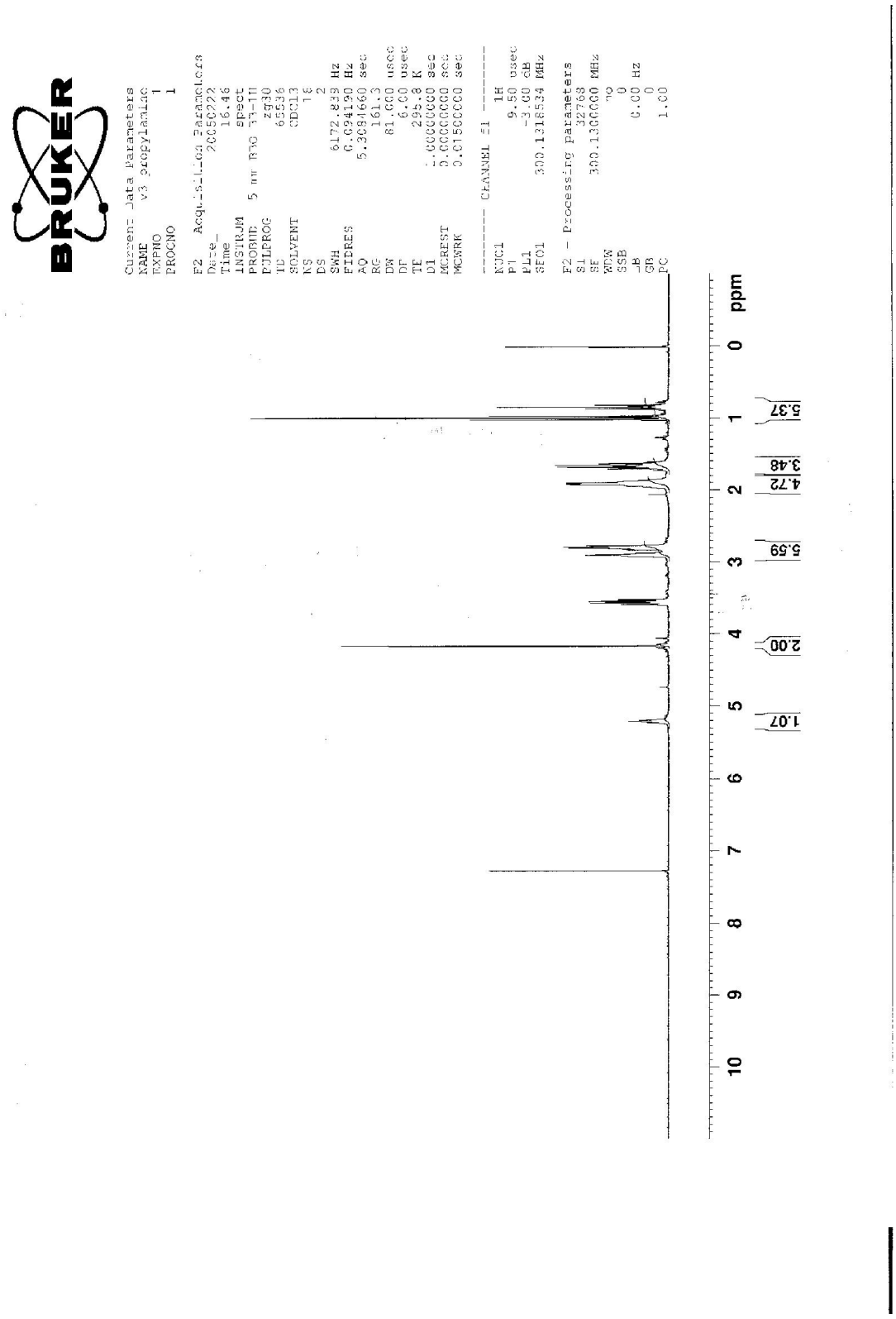

Figure A2b. Carbon 13 NMR Spectrum for **5c**.

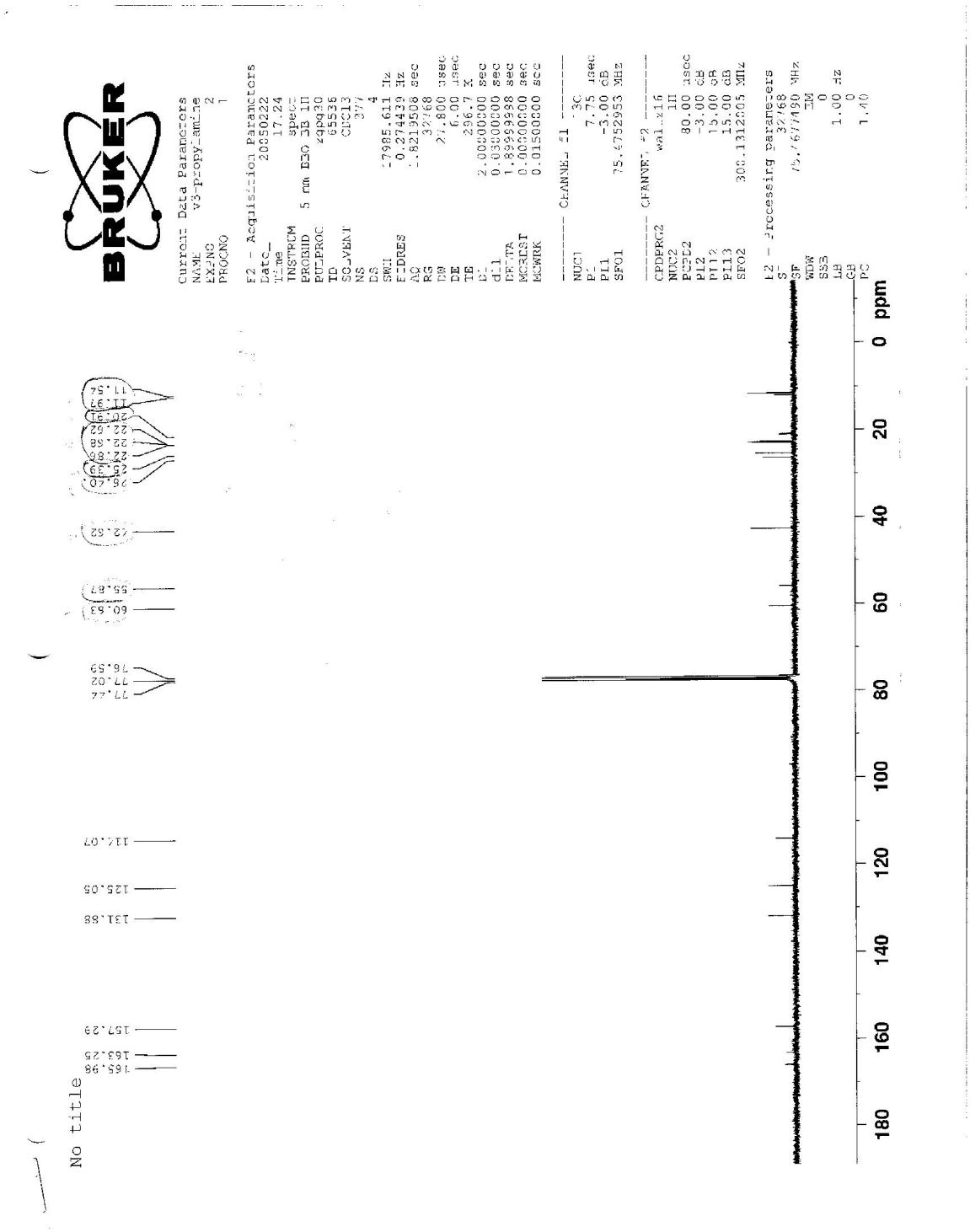

Figure A2c. Mass Spectrometry for **5c**.

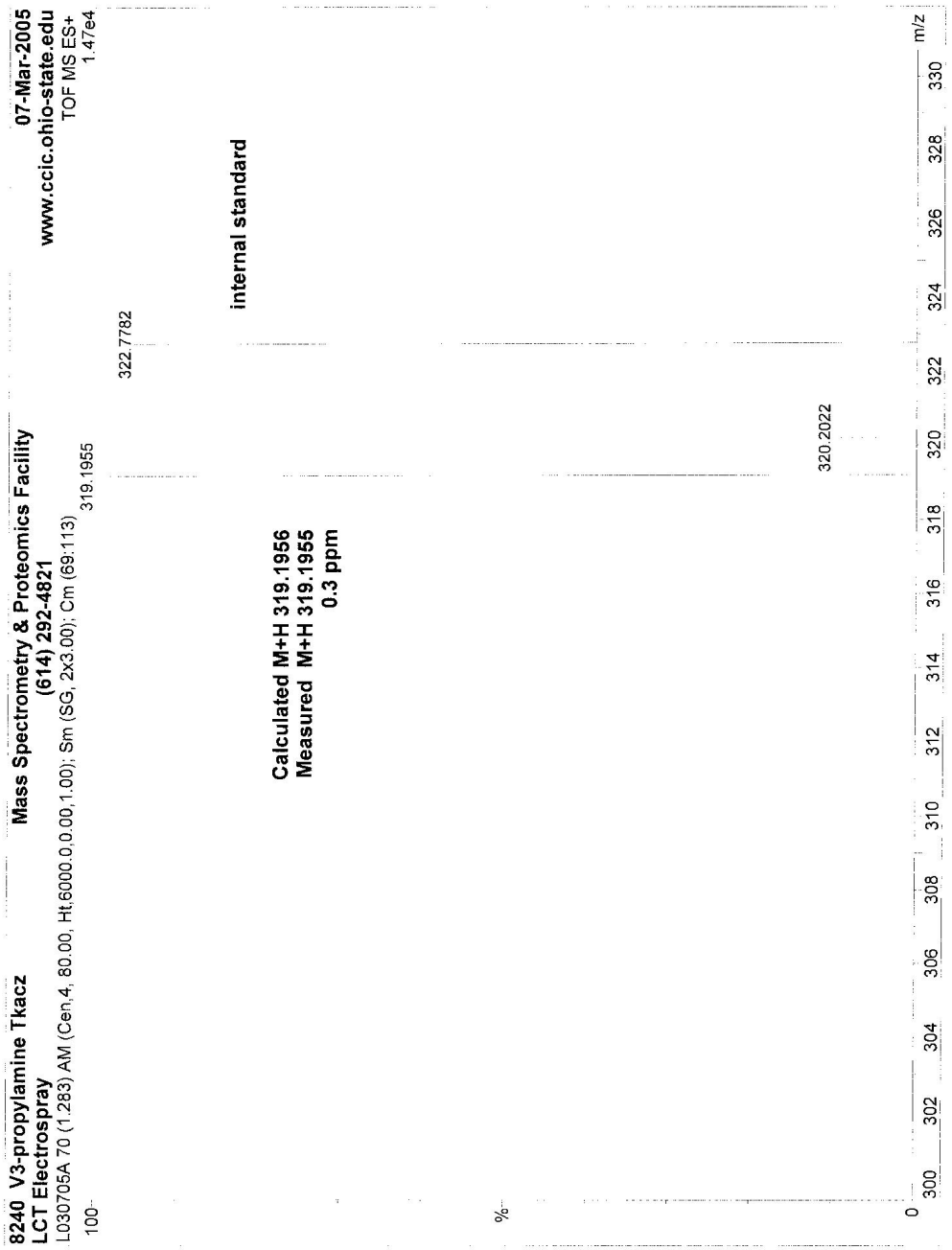

Figure A3. Proton NMR Spectrum for **5d**.

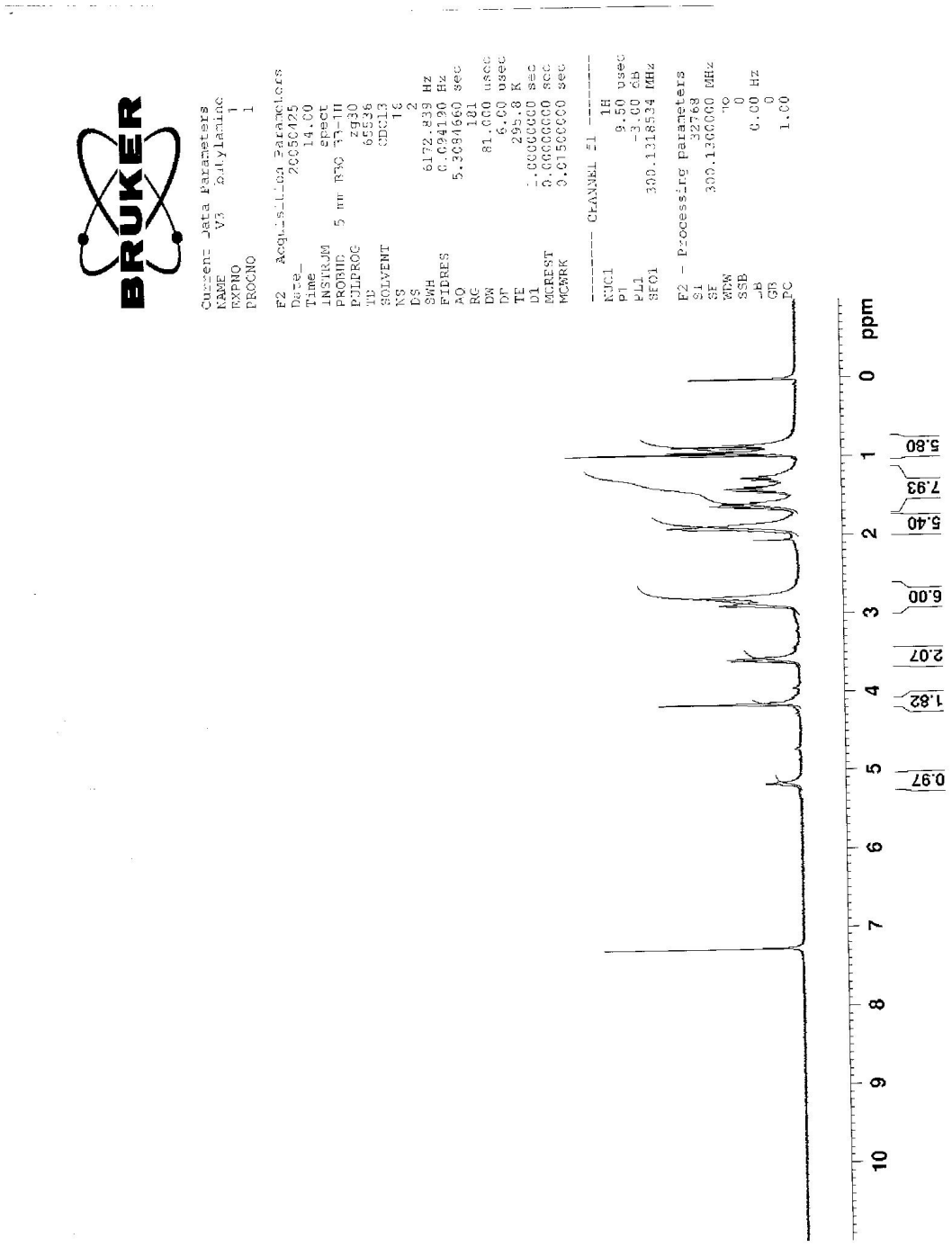

Figure A4. Proton NMR Spectrum for **5e**.